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Photoöxidation of Amino Acids in the Presence of Methylene Blue

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Observations of Raab (1) and Tappeiner and Jodlbauer (2) on the photochemical action of dyes have stimulated a great deal of research, which has been summarized in the extensive reviews of Blum (3) and Arnow (4). Most of the work has been carried out on complex sytems, such as animals or unicellular organims, which make interpretation of the results difficult. Study of the action of visible light on well-defined substances in the presence of dyes was therefore desirable. Because proteins participate in the reaction, an investigation of the photochemical action of dyes on these compounds was undertaken, and our first step in this direction was a systematic study of the amino acids. Previous work of Lieben (5), Carter (6), Harris (7), Gaffron (8), Weil and Maher (9), and Galston (10) has demonstrated the susceptibility of the aromatic amino acids to photoöxidation. The present report deals with our extended findings on the photochemical action of methylene blue on these and other amino acids.

Experimental

The manometric technique described in our previous papers (11,12) was used. To find the optimal conditions for the photoöxidation, the influence of pH was studied on the oxygen uptake of tyrosine, histidine, tryptophan, methionine, and cystine. To 1-ml. aliquots of water solutions containing 0.005 mmole/ml. of the amino acids (cystine was dissolved with the aid of a little LiOH) were added 0.5-ml. portions of 0.2 *M* phosphate buffer of pH 4.5, 5.8, 6.8, 7, 8, 8.5, and 9.2 (the last was adjusted to that pH with 0.1 *N* NaOH). In the side arm of the Warburg vessel was placed 0.5 ml. of a solution containing 0.1 mg. methylene blue. After temperature equilibrium (37°), the dye solution was tipped into the main chamber and the irradiation started as described previously (11,12). Owing to the limitation in solubility of cystine, only

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the alkaline side of the pH range was investigated. Figure 1 shows that the optimal pH for the photochemical action of methylene blue on the amino acids was about 8.5.

Photochemical Action of Methylene Blue on L-*Tyrosine and Related Compounds*

To 1-ml. samples of water solutions containing 0.005 mmole L-tyrosine, phenol, DL-alanine, glycyl-L-tyrosine, tyramine, and hordenine (*N*-dimethyltyramine) respectively, were added 0.5 ml. of 0.2 *M* phosphate buffer of pH 8.5 and 0.1 mg. methylene blue dissolved in 0.5 ml. of water. Oxygen uptake and CO_2 evolution were measured in the

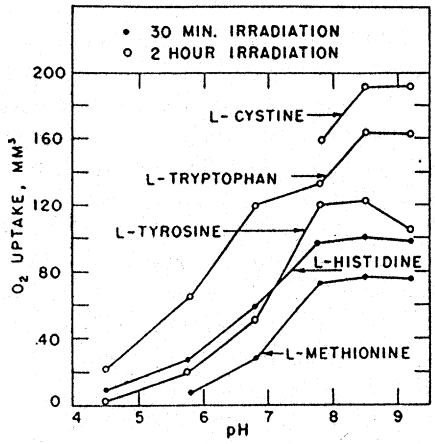

FIG. 1. Effect of pH on the photochemical action of methylene blue on tryptophan, tyrosine, histidine, methionine, and cystine.

customary fashion by placing concentrated KOH in the center well of the second vessel. To avoid retention of CO_2 by the phosphate buffer, acid was added after the completion of the reaction. In all the succeeding experiments, the conditions and substrate concentrations were the same as above unless stated otherwise. Figure 2 shows that L-tyrosine took up about 2 moles oxygen/mole amino acid. The same result obtained with glycyl-L-tyrosine (not shown in Fig. 2) indicates that the free α-amino group of tyrosine is not essential for the oxidation. The fact that alanine, which represents a portion of the tyrosine molecule, was oxidized only slightly, whereas phenol was even more reactive than tyrosine itself, indicates that the oxidation was confined only to the

aromatic nucleus. Table I shows the oxygen uptake and CO_2 evolution of these compounds during the photoöxidation. The values were obtained after a 580-min. irradiation, after which period practically no further oxygen uptake was observed.

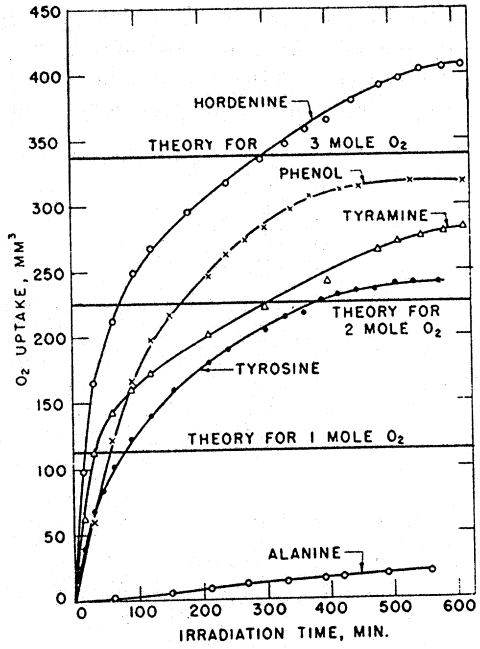

FIG. 2. Photochemical action of methylene blue on tyrosine and related compounds. Theoretical O_2 uptake calculated for 1 mole of substrate.

TABLE I

Oxygen Uptake and CO_2 Evolution of Tyrosine and Related Compounds Per Mole Produced by the Photochemical Action of Methylene Blue

	Tyrosine	Alanine	Phenol	Glycyl-tyrosine	Tyramine	Hordenine
Moles of O_2 taken up	2.14	0.12	2.81	2.17	2.51	3.64
Moles of CO_2 evolved	0.98	.13	1.05	1.02	.91	1.50

The data suggest that the CO_2 evolution originated from the phenol ring of tyrosine, thus involving rupture of the aromatic nucleus. Tyramine, which is a decarboxylation product of tyrosine, was even more reactive than tyrosine, and the fact that this compound yielded almost 1

mole of CO_2 supports our previous view that the CO_2 originates from the oxidation of the phenol portion of tyrosine and is not due to decarboxylation. The high reactivity of hordenine appears to be in agreement with our previous work (11,12), which showed the high reactivity of tertiary amino groups.

Measuring the changes in the ultraviolet absorption spectrum of tyrosine during the photochemical action of methylene blue brought additional evidence that the photoöxidation was confined to the phenol portion of tyrosine. For this purpose, a tyrosine solution was irradiated

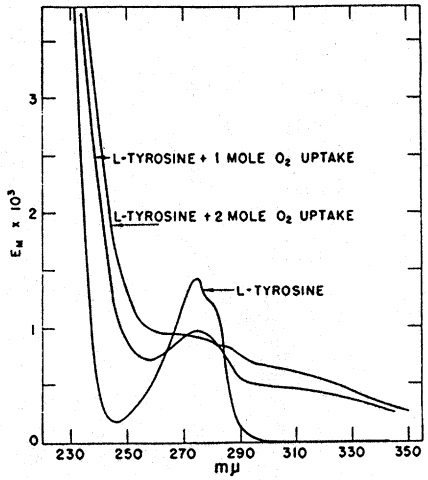

FIG. 3. Changes in the ultraviolet absorption spectrum of L-tyrosine produced by the photochemical action of methylene blue.

as described in the experiments shown in Fig. 2. After removal of the dye with a small amount of charcoal, the filtrate was investigated in 0.1 *N* HCl solution. Slight losses during the charcoal treatment were determined by total N determinations. The absorption curves (Fig. 3) show that a marked change occurred when 1 mole of tyrosine took up 1 mole of O_2. The absorption maximum at 274 mμ dropped considerably, and the minimum shifted from 246 mμ to 258. At 2 moles of O_2 uptake, both the absorption maximum and minimum disappeared, indicating elimination of the phenol ring.

During the photochemical action of methylene blue on tyrosine, no melanin formation was observed.

NH_3 Liberation. Using the Conway diffusion technique (13), we could demonstrate no NH_3 liberation during the photoöxidation of tyrosine. The course of the photoöxidation of tyrosine was followed by the Bernhart modification (14) of the Millon-Weiss color reaction. A progressive decrease in tyrosine was observed, with complete disappearance of color when 2 moles of oxygen had been taken up.

Photochemical Action of Methylene Blue on L-*Histidine and Related Compounds*

One-ml. samples of L-histidine, imidazole, DL-alanine, and histamine solutions were irradiated as described in experiments presented in Fig.

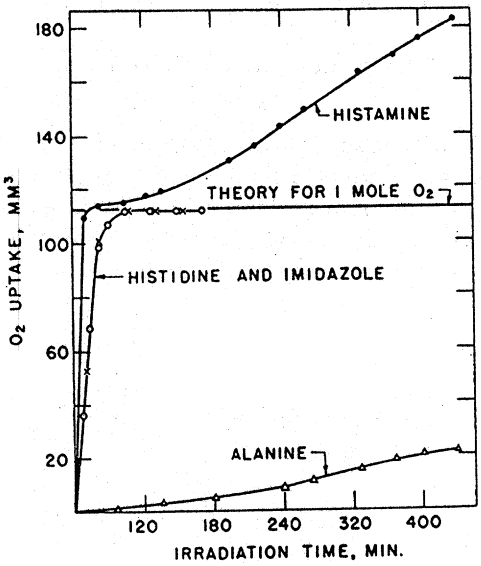

FIG. 4. Photochemical action of methylene blue on histidine and related compounds. Theoretical O_2 uptake calculated for 1 mole of substrate.

2. The results (Fig. 4) indicate that the entire oxygen uptake of histidine was due to the imidazole ring of the histidine molecule, in analogy to the oxidation of the phenol ring in the case of tyrosine. In contrast to tyrosine, however, there was no CO_2 evolution. Histamine, which is a decarboxylation product of histidine, took up 1.88 moles of oxygen and evolved 0.66 mole CO_2 during the photoöxidation. The distinct

inflection in the oxygen uptake curve at the point where the uptake reached 1 mole of oxygen indicates an additional reaction to the one observed for histidine and imidazole.

Destruction of histidine during the photoöxidation was followed by the Macpherson modification (15) of the Pauly reaction. Photoöxidation was accompanied by progressively less color production, with complete disappearance of histidine at 1 mole of oxygen uptake.

Ammonia, amounting to 3.8% of the total nitrogen, was liberated during the reaction.

There is preliminary evidence that during the photoöxidation of histidine, rupture of the imidazole ring takes place, with formation of an aldehyde group, which then might form an internal Schiff base with the α-amino group or with the α-amino group of a second molecule.

Photochemical Action of Methylene Blue on L-*Tryptophan and Related Compounds*

L-Tryptophan, indole, and DL-alanine (0.0025 mmole/ ml.) were irradiated as described for experiments presented in Fig. 2. Figure 5 shows that L-tryptophan and indole took up almost 4 moles of oxygen, whereas alanine, which constitutes the aliphatic side chain of the tryptophan molecule, took up only 0.14 mole. As in the case of tyrosine and his-

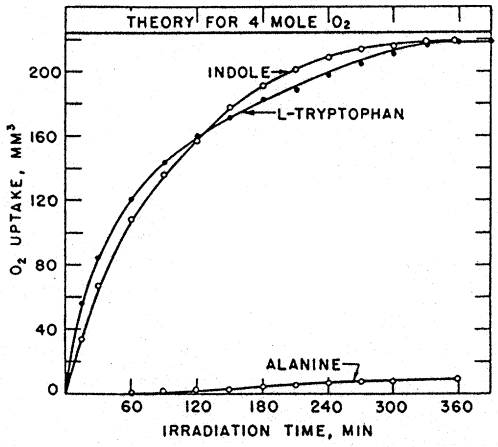

FIG. 5. Photochemical action of methylene blue on tryptophan and related substances. Theoretical O_2 uptake calculated for 1 mole of substrate.

tidine, the entire oxygen uptake appears to be limited to the aromatic portion of tryptophan, namely, to indole. This assumption was supported by the fact that the CO_2 evolution during the photoöxidation of tryptophan was 1.93 moles, whereas indole under the same circumstances gave a value of 2.05 moles of CO_2.

Measuring changes in the ultraviolet absorption spectrum of tryptophan during the photochemical action of methylene blue supplied addi-

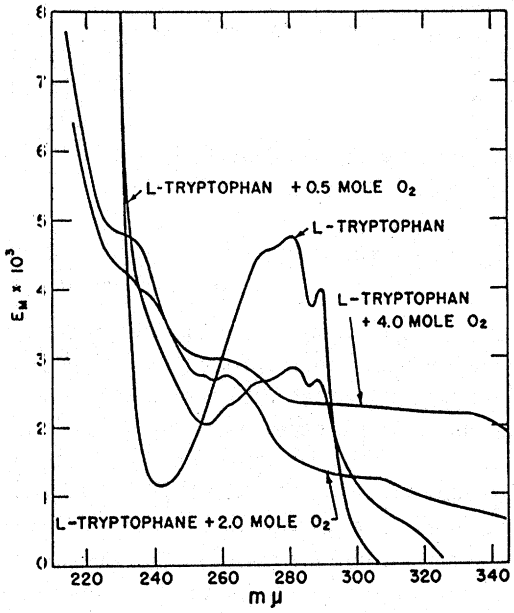

FIG. 6. Changes in the ultraviolet absorption spectrum of L-tryptophan produced by the photochemical action of methylene blue.

tional evidence that the indole portion of this amino acid was involved in the oxidation. The procedure described for tyrosine (Fig. 3) was used. However, because of a slight opalescence of the irradiated tryptophan, 57% ethanol was used as solvent. Figure 6 shows that even at 0.5 mole of oxygen uptake, a marked drop in the absorption maximum at 280 mμ could be observed, and the original absorption minimum shifted from 242 to 255 mμ. At 2 moles of oxygen uptake/mole tryptophan the absorption maximum at 280 mμ, which is due to conjugation of the ethylenic linkage in the pyrrole ring with the benzene chromo-

phore, was wiped out entirely. The formation of a new maximum at 260 mμ indicates that the photoöxidation at this point was confined to the pyrrole ring, inasmuch as the benzene chromophore alone should have a maximum at about 260 mμ. At 4 moles of oxygen uptake, no minimum or maximum could be observed. Whether the benzene chromophore was photoöxidized at this point could not be decided from the absorption curve obtained. The resistance of phenylalanine to photooxidation, as shown in Table V, however, indicates that this ring was not involved in the reaction. By following the decrease of tryptophan during the photoöxidation by the Shaw and McFarlane method (16), progressive disappearance of this amino acid was observed. At 2 moles of oxygen uptake/mole tryptophan, the color reaction was negative, even though maximal oxygen uptake was 4 moles. The NH_3 liberation measured by the Conway technique amounted to 16.1% of the total nitrogen.

Photochemical Action of Methylene Blue on Methionine and Its Derivatives

Aqueous solutions of methionine, allocystathionine,[2] *N*-acetylmethionine,[2] γ-methylmercaptobutyric acid,[2] dehydromethionine,[2] methionine sulfoxide,[2] and nicotinyl methionine amide[3] were irradiated as described for experiments presented in Fig. 2. The results plotted in Fig. 7 show that elimination of the amino group (as in γ-methylmercaptobutyric acid), substitution of the amino group (as in *N*-acetylmethionine), or substitution of both amino and carboxyl groups (as in nicotinyl methionine amide) in methionine did not prevent the photochemical action of methylene blue on these compounds. The photooxidation of γ-methylmercaptobutyric acid was identical to that of methionine in both rate and extent. With acetylmethionine and nicotinyl methionine amide a break in the reaction rate curve at about 0.65 mole oxygen uptake was observed but the total oxygen uptake was not affected. Allocystathionine took up 1.47 moles oxygen/mole substrate, presumably because of the replacement of the methyl group by the 2-amino-2-carboxyethyl group. Lavine's dehydromethionine (17) and

[2] We gratefully acknowledge receipt of these samples from Drs. T. F. Lavine and N. F. Floyd, Institute of Cancer Research, Philadelphia, Pa.

[3] Obtained through the courtesy of Dr. C. G. Niemann, California Institute of Technology, Pasadena, Calif.

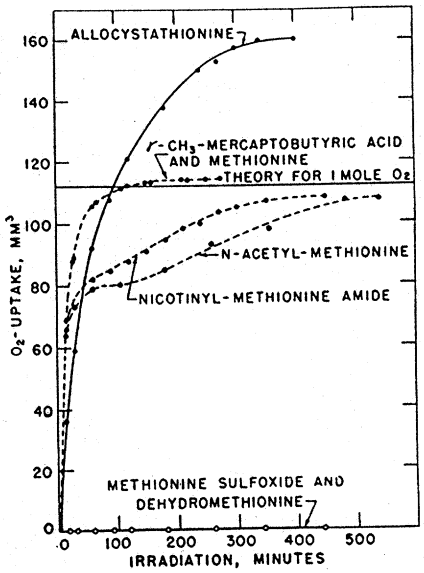

FIG. 7. Photochemical action of methylene blue on L-methionine and its derivatives. Theoretical O_2 uptake calculated for 1 mole of substrate.

methionine sulfoxide did not react. Carbon dioxide was not produced in any of these experiments.

Photochemical Action of Methylene Blue on L-*Cystine and Related Compounds*

In these experiments, L-cystine was used instead of cysteine, since the latter was rapidly converted to cystine by atmospheric oxygen alone. A solution was prepared by dissolving the cystine in water and LiOH until pH 8.5 was reached. One-ml. aliquots of solution containing 0.005 mmole L-cystine, 0.005 mmole cystine disulfoxide, and 0.01 mmole cysteic acid, respectively, were irradiated as described for experiments presented in Fig. 2, with the results shown in Fig. 8. The reactivities of cystine disulfoxide and cysteic acid indicate that these compounds might be considered as intermediary oxidation products of cystine. Table II summarizes the oxygen uptake and CO_2 evolution of these compounds, obtained after a 420-min. irradiation, after which period practically no further oxygen uptake was observed.

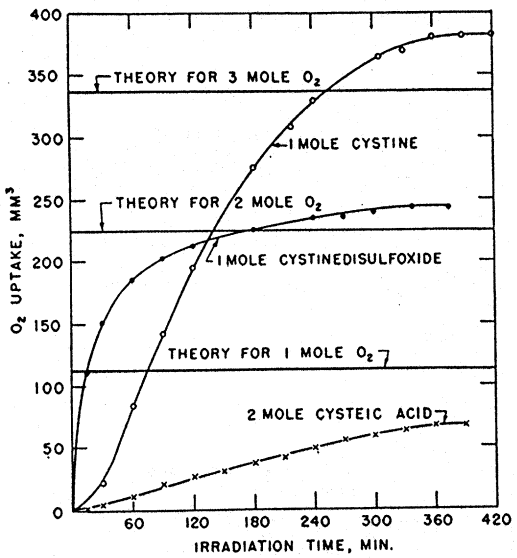

FIG. 8. Photochemical action of methylene blue on cystine and its derivatives. Theoretical O_2 uptake calculated for 1 mole of substrate.

TABLE II

Oxygen Uptake and CO_2 Evolution of Cystine and Related Compounds Produced by the Photochemical Action of Methylene Blue

	Cystine, 1 mole	Cystine disulfoxide, 1 mole	Cysteic acid, 2 moles
Moles of O_2 taken up	3.41	2.19	0.60
Moles of CO_2 evolved	1.96	1.54	0.39

During the photoöxidation of cystine, 25.8% of the sulfur was converted to sulfate, determined as $BaSO_4$. No liberation of NH_3 could be demonstrated by the Conway technique.

Peroxide Formation During the Photochemical Action of Methylene Blue on Tyrosine, Histidine, Tryptophan, Methionine and Cystine

In our previous work on the photochemical action of methylene blue on nicotine (11,12), it was shown that the first step in the reaction was a dehydrogenation of nicotine accompanied by a simultaneous hydrogenation of the dye. The reoxidation of the dye resulted in the formation of H_2O_2, which in turn reacted with the dehydrogenated nicotine. If the photoöxidation was carried out in the presence of sodium pyru-

vate, the H_2O_2 produced as an intermediate reacted with the pyruvate, yielding 1 mole of acetate and 1 mole of CO_2/mole peroxide. An increase in CO_2 evolution during the photoöxidation of amino acids under similar conditions, therefore, would be indicative of the formation of H_2O_2 or organic peroxide. To 1-ml. aliquots of water solutions containing 0.005 mmole L-tyrosine, L-histidine, DL-methionine, and L-cystine and 0.0025 mmole L-tryptophan, respectively, were added 0.5-ml. portions of a solution containing 0.013 mmole Na pyruvate. The solutions were irradiated as described for experiments presented in Fig. 2. The results in Table III show that during the photochemical action of methylene

TABLE III

Oxygen Uptake and CO_2 Evolution of Tyrosine, Histidine, Tryptophan, Methionine and Cystine Per Mole Produced by the Photochemical Action of Methylene Blue in the Presence of Na Pyruvate

	O_2 taken up	CO_2 evolved		O_2 taken up	CO_2 evolved
	moles	*moles*		*moles*	*moles*
MB[a] + Na pyruvate	0.0	0.0	Histidine + MB	1.00	0.0
Tyrosine + MB	2.05	1.01	Histidine + MB + Na pyruvate	1.02	.06
Tyrosine + MB + Na pyruvate	2.03	2.10	Methionine + MB	1.00	.0
Tryptophan + MB	3.98	1.90	Methionine + MB + Na pyruvate	1.02	1.04
Tryptophan + MB + Na pyruvate	4.08	3.86	Cystine + MB	3.41	1.96
			Cystine + MB + Na pyruvate	3.70	2.20

[a] Methylene blue.

blue on tyrosine and methionine, 1 mole of H_2O_2 or organic peroxide was formed per mole of amino acid, as indicated by the additional CO_2 evolution. In the case of tryptophan the formation amounted to an additional 2 moles/mole amino acid, but in histidine and cystine the lack of additional CO_2 evolution indicates the absence of peroxide formation.

The presence of H_2O_2 or organic peroxides at the end of the photoöxidation of tyrosine, histidine, tryptophan, methionine, and cystine was investigated. The experiments were made in duplicate in the manner described for experiments presented in Table III, except that Na pyruvate was omitted. After the photoöxidation of these amino

acids was completed, 0.5 ml. of a highly purified catalase preparation, and in the case of the duplicate, 0.5 ml. of an aqueous MnO_2 suspension containing 2 mg., were added from the side arm of the Warburg vessel. Any formation of H_2O_2 should result in the evolution of 0.5 mole O_2/mole H_2O_2 by the action of catalase. Only in methionine could we demonstrate the formation of H_2O_2 after the completion of the photo-oxidation of these amino acids, although the experiments carried out in the presence of Na pyruvate (Table III) indicated the formation of an intermediary oxidizing agent also in tyrosine and tryptophan. The oxygen evolved by methionine after addition of catalase was 0.46 mole, and the addition of MnO_2 resulted in the formation of 0.48 mole. In irradiated methionine solutions the following qualitative tests for hydrogen peroxide were also found to be strongly positive:

(*a*) Luminescence with 3-aminophthalhydrazide.
(*b*) Yellow color with cerium sulfate.
(*c*) Blue color with sulfuric acid–potassium dichromate.
(*d*) Iodine liberation from iodide.

The fact that only methionine yielded analytically detectable amount of H_2O_2 may indicate that in tyrosine and tryptophan this intermediary oxidizing agent (H_2O_2 or organic peroxide) was used up during the photoöxidation.

Characterization of the Product from Methionine Irradiation

Five grams DL-methionine and 15 mg. methylene blue were dissolved in 250 ml. water in a three-necked flask equipped with a mercury-seal stirrer and reflux condenser. The flask was placed in a cylindrical glass water bath kept at 37°. A slow stream of air was provided through the condenser during the irradiation. As a light source a 300-w. spotlight tungsten lamp, placed 10 cm. below the reaction vessel, was used. Irradiation and stirring for a period of 1.5 hr. brought the reaction to completion as shown by a negative test for methionine by the color reaction of McCarthy and Sullivan (18). The reaction mixture was then treated with activated carbon (Norit), filtered, and evaporated at 50° *in vacuo*. The white amorphous residue (5.45 g.) was dissolved in hot 50% ethanol. The solution was cooled to room temperature and acetone was added until the solution became turbid. Cooling in the refrigerator produced a crystalline precipitate which weighed 4.62 g.

After repeated recrystallization the substance melted at 223.8–225.0° with gas evolution. A mixed melting point with an authentic sample of methionine sulfoxide[2] gave no depression.

Anal. Calc. for $C_5H_{11}NSO_3$: C, 36.36; H, 6.71; N, 8.48; S, 19.37%. Found: C, 36.22; H, 6.90; N, 8.29; S, 19.12%.

A crystalline derivative of authentic methionine sulfoxide prepared with 1-chloro-2,4-dinitrobenzene by the method of Sanger (19) melted at 189.6–191.8°; a similar derivative of the irradiation product melted at 189.4–191.0°; mixed m.p., 189.8–191.8°.

It is evident from these data that methionine sulfoxide is the end product of the photochemical action of methylene blue on methionine.

Reaction Mechanism of Methionine Irradiation

A reaction mechanism for the conversion of methionine to its sulfoxide in these experiments must account for (*a*) an uptake of 1 mole oxygen/mole methionine and (*b*) the formation of 1 mole hydrogen peroxide/mole amino acid. The over-all reaction $C_5H_{11}NSO_2 + H_2O + O_2 = C_5H_{11}NSO_3 + H_2O_2$ is in accord with these facts but neglects the indispensability of methylene blue for the photoöxidation. Since irradiation of methionine and methylene blue in the absence of oxygen results in a slow bleaching of the dye, a dehydrogenation of methionine apparently occurs, and it is possible that a similar dehydrogenation takes place in the presence of oxygen as the first step in the photoöxidation.

That methionine is readily dehydrogenated under certain conditions was shown by Lavine (17,20). The addition of iodine to a solution of methionine at pH 7 effected a dehydrogenation of the amino acid, and the reaction was reversed on acidification. Lavine's methods for the quantitative determination of both methionine (20) and the dehydrogenation product, dehydromethionine (21), are based on these observations. We investigated the possible formation of dehydromethionine in our experiments by applying Lavine's methods during the course of photoöxidation. In addition to determinations of methionine and dehydromethionine, the formation of methionine sulfoxide was followed by the iodometric method of Toennies and Kolb (22). A 1% solution of DL-methionine containing 0.005% methylene blue was irradiated in the apparatus described for the preparation of the sulfoxide. At the beginning of the irradiation and periodically thereafter, 10-ml. aliquots of the solution were removed, decolorized with Norit, and filtered. The

filtrate was treated with a drop of a highly purified horse liver catalase solution to eliminate hydrogen peroxide. The absence of hydrogen peroxide was assured by testing for luminescence with 3-aminophthalhydrazide (23). One-ml. aliquots were then used for the iodometric determinations of methionine, dehydromethionine, and methionine sulfoxide. In all experiments performed, irradiation resulted in a progressive decrease in methionine with an approximately equivalent conversion to methionine sulfoxide. The oxidation was essentially complete in about 90 min. The formation of dehydromethionine was quite variable. In a number of experiments dehydromethionine could be detected only in traces, while in others it accumulated gradually. The data for a representative positive experiment are summarized in Table IV. A maximal value of 17% dehydromethionine was attained in one experiment. With the thought that hydrogen peroxide produced during the irradiation

TABLE IV

Photochemical Action of Methylene Blue on Methionine and the Formation of Dehydromethionine and Methionine Sulfoxide

Time of irradiation	Methionine[a]	Dehydro-methionine	Methionine sulfoxide[a]
min.	%	%	%
0	100	0	0
30	60.8	2.4	38.6
120	7.5	8.4	99.4

[a] Titrations would include any dehydromethionine formed; see text.

might oxidize dehydromethionine, several irradiations were carried out in the presence of manganese dioxide or sodium pyruvate. The results were no different in these cases. It should be mentioned that the methods of analysis employed for methionine and methionine sulfoxide were not entirely specific. For example, the methionine titration itself involves conversion to dehydromethionine and would, therefore, measure not only methionine but dehydromethionine. Also the sulfoxide titration would determine not only sulfoxide but any dehydromethionine present as well. Fortunately, the conditions for determination of dehydromethionine are specific for the purpose intended, and the results obtained should represent values due to the dehydrogenation product. The reason for our variable success in demonstrating the dehydrogenation of methionine, however, requires further investigation.

To secure additional evidence for the formation of dehydrogenated methionine as an intermediate during irradiation, changes in the ultraviolet absorption spectrum were measured. Conditions for irradiation were as just described. Methylene blue was removed with Norit, and the colorless filtrates were used for measurements of the absorption spectra before irradiation and after photoöxidation was completed. The ratio of dehydromethionine to methionine sulfoxide in this particular photoöxidized solution was 8:92 as measured iodometrically. The results are plotted in Fig. 9 (curve 2, methionine; curve 4, photooxidized solution). Also shown are the spectra of Lavine's dehydro-

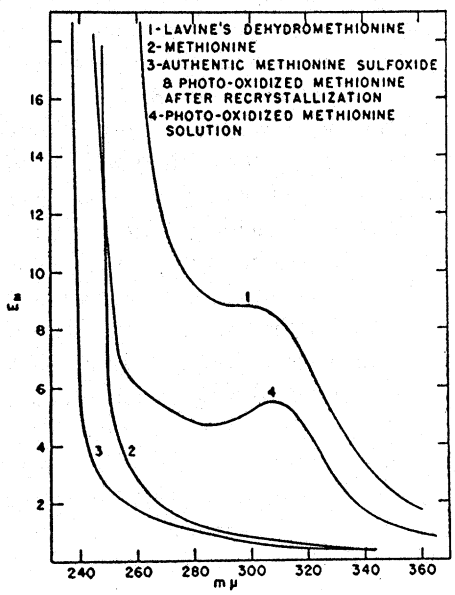

FIG. 9. Ultraviolet absorption of methionine derivatives.

methionine (curve 1), an authentic specimen of methionine sulfoxide (curve 3), and methionine sulfoxide isolated from the photoöxidized solution and purified by recrystallization (curve 3). Neither the original methionine solution nor the samples of methionine sulfoxide showed any characteristic absorption, but the photoöxidized methionine solution gave a distinct maximum at 308 mμ and a minimum at 285 mμ.

The substance responsible for the maximum and minimum is presumably a dehydrogenated methionine which is readily removed from the sulfoxide during isolation and recrystallization of the sulfoxide. However, the substance cannot be Lavine's dehydromethionine since the latter shows only an inflection at about 300 mμ in its absorption curve. It should be emphasized that the photoöxidized solution contains but 8 parts dehydrogenation product in the presence of 92 parts sulfoxide. The molar extinction coefficients plotted in curve 4 are therefore composite, and it may be inferred that the characteristic features of the curve would have been much more pronounced had it been possible to deal with more concentrated solutions of dehydrogenation product. Since this was not possible, a calculation of molar extinction coefficients for the pure dehydrogenation product was made from the data in curves 3 and 4. The values obtained for the hypothetical intermediate were about 61 at 308 mμ and about 51 at 285 mμ.

The exact nature of this dehydrogenation product is uncertain. It appears to resemble Lavine's dehydromethionine (2) in that it liberates iodine from an acidified solution of potassium iodide. Further, because of the substantial variations in yields of dehydrogenation product, which we were unable to correlate in any way with changes in experimental conditions during photoöxidation, we conclude that the intermediate is unstable and that it is converted readily to sulfoxide. In this respect, too, the intermediate is similar to Lavine's dehydromethionine. On the other hand, our dehydrogenation product differs from Lavine's in ultraviolet absorption. One might speculate on the significance of the absorption maximum of our dehydrogenation product in connection with the studies of the ultraviolet absorption of dehydropeptides discussed by Greenstein (24). However, such speculation seems to be unwarranted at this time.

Photochemical Action of Methylene Blue on the Other Amino Acids

All the amino acids discussed so far are characterized by their high degree of photoreactivity. The amino acids presented in Table V acted sluggishly or not at all. Experimental conditions were the same as described for experiments presented in Fig. 2. The results in Table V represent values obtained after 480 min. irradiation, at which point practically no further oxygen uptake could be observed.

TABLE V

Oxygen Uptake and CO_2 Evolution of Various Amino Acids Per Mole Produced by the Photochemical Action of Methylene Blue

Amino acid	O_2 taken up/ mole	CO_2 evolved/ mole	Amino acid	O_2 taken up/ mole	CO_2 evolved/ mole
	mole	*mole*		*mole*	*mole*
Glycine	0.11	0.10	DL-Phenylalanine	0.0	0.0
DL-Alanine	0.17	0.15	L-Proline	0.32	0.30
DL-Valine	0.18	0.19	DL-Aspartic acid	0.20	0.20
DL-Leucine	0.15	0.16	L-Glutamic acid	0.26	0.24
DL-Serine	0.17	—	L-Lysine	0.20	0.15
DL-Threonine	0.19	0.19	L-Arginine	0.13	0.06

Does the Peptide Bond Participate in the Photochemical Action of Methylene Blue?

One ml. of water solutions of glycine, glycylglycine, tetraglycine, and hexaglycine[4] were irradiated as described for experiments presented in Fig. 2. Table VI shows that the increase of peptide bonds did not increase the oxygen uptake and indicates that these bonds do not participate in the photochemical action of methylene blue.

TABLE VI

Oxygen Uptake of Glycine and Its Peptides Per Mole Produced by the Photochemical Action of Methylene Blue

	Glycine	Glycyl-glycine	Tetra-glycine	Hexa-glycine
Mole of O_2 taken up	0.16	0.18	0.15	0.19

Photochemical Action of Methylene Blue on L-Lysine, α-N-CH_3-lysine and α-N-Di-CH_3-lysine

As reported in our previous work (11,12), the photochemical action of methylene blue was greatly increased when a primary amino group was converted into a secondary amino group, and more so when the tertiary derivative was formed. To test the validity of this observation, 1-ml. aliquots of water solutions of L-lysine, α-*N*-CH_3-lysine, and α-*N*-di-CH_3-lysine were irradiated as described for experiments presented in Fig. 2. The results in Fig. 10 show clearly the increased photoreactivity of the mono derivative over lysine and the further increase in reactivity of the disubstituted lysine.

[4] The glycine peptides were received from Dr. E. F. Mellon of this laboratory.

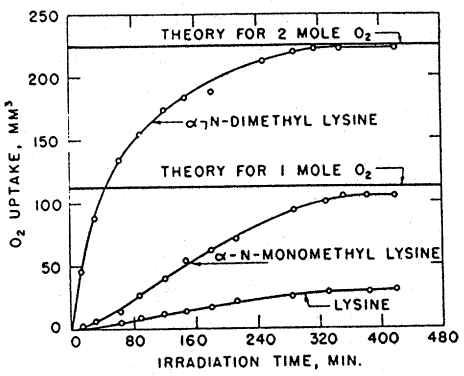

FIG. 10. Photochemical action of methylene blue on lysine and its derivatives. Theoretical O_2 uptake calculated for 1 mole of substrate.

DISCUSSION

The photochemical action of dyes on amino acids has been studied by various workers (5–10). There appears to be general agreement that tryptophan, tyrosine, and histidine are particularly sensitive to the photochemical action of dyes. In addition to these amino acids, methionine (9,10) and cystine are also susceptible to the photoöxidation. The rest of the amino acids investigated acted sluggishly or not at all.

In the case of tyrosine, tryptophan and histidine, it has been shown that the observed oxygen uptake and CO_2 evolution are due to the cyclic nucleus of these amino acids, involving rupture of the ring system. Methionine, which takes up 1 mole oxygen/mole amino acid during the photoöxidation, is converted to methionine sulfoxide; cystine is oxidized probably beyond the cysteic acid stage.

Our experiments on the photoöxidation of amino acids in the presence of pyruvate have shown that the reaction may proceed in different ways with different amino acids. Addition of pyruvate to histidine and cystine did not produce additional CO_2 during photoöxidation and, therefore, H_2O_2 is apparently not formed as an intermediate. With tyrosine and tryptophan additional CO_2 was evolved; and methionine, which in the absence of pyruvate did not evolve CO_2, produced CO_2 in its presence. Thus, the photoöxidation of tyrosine, tryptophan, and methionine may involve a dehydrogenation and subsequent H_2O_2 or organic peroxide formation (25). The oxidizing agent can be used up, as with tyrosine and tryptophan, leading to rupture of the aromatic

nucleus, or it may accumulate as H_2O_2, as in the case of methionine. Our evidence indicates that such a dehydrogenation, which is considered to be the first step in the photochemical action of methylene blue, actually occurs in methionine.

The data for methionine, though not conclusive, are in accord with the following reaction mechanism for the conversion to sulfoxide:

$$\text{Methionine} + \text{MB} = (\text{dehydrogenated methionine}) + \text{leuco MB}$$
$$\text{Leuco MB} + O_2 = \text{MB} + H_2O_2$$
$$\text{Dehydrogenated methionine} + H_2O = \text{methionine sulfoxide}$$

Methylene blue being an oxidation–reduction indicator, any hydrogen transferred to this dye during the photoöxidation should be manifested by its bleaching if anaerobic conditions are used. Such a rapid hydrogen transfer in nicotine (11,12) has been reported. In amino acids, however, no bleaching of the dye takes place within short periods, and only extended irradiation (about 6 hr.) produces visible bleaching. It is possible that equilibrium between the amino acid and methylene blue exists, by which hydrogen transfer from the former to the latter proceeds only to a limited extent. Reoxidation by atmospheric oxygen of the leuco-dye produced, however, upsets this equilibrium, and further hydrogen transfer would be necessary to restore it. Reactions of a similar nature were observed by Windaus and Borgeaud (26), Windaus and Brinken (27), and Carter (6) during irradiation of ergosterol and tyrosine.

Summary

A systematic study was made of the photochemical action of methylene blue on amino acids.

Tyrosine, tryptophan, histidine, methionine, and cystine were highly reactive during the photoöxidation; the rest of the amino acids acted sluggishly or not at all.

In tyrosine, tryptophan, and histidine, the entire oxygen uptake and CO_2 evolution were due to the cyclic nucleus, involving rupture of the rings.

During the photochemical action of methylene blue on tyrosine, tryptophan, and methionine, intermediary oxidizing agents were formed; in methionine this was shown to be H_2O_2.

The photoöxidation of methionine resulted in the formation of methionine sulfoxide as an end product.

Iodometric titration and measurement of ultraviolet absorption during irradiation of methionine indicate the formation of an intermediary dehydrogenation product which appears to differ from Lavine's dehydromethionine.

Cystine was photooxidized, probably beyond the cysteic acid stage.

Peptide bonds did not participate in the photochemical action of methylene blue.

Methylation of the α-amino group of lysine to the corresponding secondary and tertiary compounds produced increased reactivity in the photooxidation.

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